

- 73 -

CLAIMS

1. A method for the production of maize seeds homozygous for a transgene conferring artificial  
5 nuclear male sterility ("AMS") and heterozygous for a fertility-restoring gene linked to a "small seed" phenotype marker, comprising the steps consisting in:

10 a) crossing a male sterile young maize plant heterozygous for the AMS transgene with a fertility-restoring young maize plant comprising in its genome a fertility-restoring gene linked to a "small seed" phenotype marker,

15 b) selecting, by means of the "small seed" phenotype, the maize seeds comprising in their genome a fertility-restoring gene linked to a "small seed" phenotype marker,

20 c) self-fertilizing the young maize plants derived from seeds selected according to step b),

25 d) selecting the seeds homozygous for the AMS transgene and heterozygous for the fertility-restoring gene linked to a "small seed" phenotype marker.

2. A method for the production of maize seeds homozygous for a transgene conferring artificial  
30 nuclear male sterility ("AMS") and heterozygous for a fertility-restoring gene linked to a "small seed" phenotype marker, comprising the steps consisting in:

35 a) crossing a male sterile young maize plant heterozygous for the AMS transgene with a fertility-restoring young maize plant comprising in its genome a fertility-restoring gene linked to a "small seed" phenotype marker,

- b) genotyping the seeds obtained by means of the cross according to step a),
- 5 c) self-fertilizing the young maize plants derived from the seeds genotyped according to step b),
- d) selecting the seeds homozygous for the AMS transgene and heterozygous for the fertility-restoring gene linked to a "small seed" phenotype marker.

3. A maize seed homozygous for an AMS transgene and heterozygous for a fertility-restoring gene linked to a "small seed" phenotype marker, which can be obtained by the method as claimed in claim 1 or 2.

4. A method for the production of maize seeds homozygous for a transgene conferring artificial nuclear male sterility ("AMS"), comprising the steps consisting in:

- a) crossing a male sterile young maize plant heterozygous for the AMS transgene with a fertility-restoring young maize plant comprising in its genome a fertility-restoring gene linked to a "small seed" phenotype marker,
- 25 b) selecting, by means of the "small seed" phenotype, the maize seeds comprising in their genome a fertility-restoring gene linked to a "small seed" phenotype marker,
- 30 c) self-fertilizing the young maize plants derived from the seeds selected according to step b),
- 35 d) selecting seeds homozygous for the AMS transgene and heterozygous for the fertility-

restoring gene linked to a "small seed" phenotype marker,

5 e) self-fertilizing young maize plants derived from seeds according to step d),

f) selecting seeds homozygous for the AMS transgene.

10 5. A method for the production of maize seeds homozygous for a transgene conferring artificial nuclear male sterility ("AMS"), comprising the steps consisting in:

15 a) crossing a male sterile young maize plant heterozygous for the AMS transgene with a fertility-restoring young maize plant comprising in its genome a fertility-restoring gene linked to a "small seed" phenotype marker,

20 b) genotyping the seeds obtained by means of the cross according to step a),

25 c) self-fertilizing the young maize plants derived from the seeds genotyped according to step b),

30 d) selecting the seeds homozygous for the AMS transgene and heterozygous for the fertility-restoring gene linked to a "small seed" phenotype marker,

e) self-fertilizing young maize plants derived from seeds according to step d),

35 f) selecting seeds homozygous for the AMS transgene.

6. A method for the production of maize seeds homozygous for an AMS transgene, comprising the steps

consisting in:

a) self-fertilizing young maize plants derived from seeds as claimed in claim 3,

5

b) selecting seeds homozygous for an AMS transgene.

7. The method as claimed in one of claims 1, 2 and 4 to 6, characterized in that at least one selection step comprises densimetric separation.

8. The method as claimed in claim 7, characterized in that the densimetric separation is carried out using a densimetric table.

9. A method for the production of a seed heterozygous for an AMS transgene, comprising the crossing of a young maize plant derived from a seed homozygous for an AMS transgene, which can be obtained by the method as claimed in one of claims 4 to 8, with a young maize plant having a wild-type genotype.

10. A method for the production of a seed heterozygous for an AMS transgene, characterized in that the method as claimed in one of claims 4 to 8 also comprises the crossing of a young maize plant derived from said seed homozygous for an AMS transgene, with a young maize plant having a wild-type genotype.

30

11. The method as claimed in one of claims 1, 2 and 4 to 10, in which the AMS transgene conferring artificial nuclear male sterility is the barnase gene, which is included in an expression cassette, under the control of a promoter specific for pollen generation, in particular an anther-specific promoter such as pA3, pA6, pA9, pTA29, or of the Mac2 promoter, and of the CaMV 3' or Nos 3' terminator, genetically linked to a gene encoding a selection agent under the control of

the actin promoter-actin intron and of the CaMV 3' or Nos 3' terminator.

12. The method as claimed in claim 11, characterized  
5 in that the expression cassette comprising the barnase gene also comprises a gene encoding a protein of therapeutic and/or prophylactic interest genetically linked to the barnase gene.

10 13. The method as claimed in claim 11 or 12, characterized in that said promoter is the pA9 promoter specific for pollen generation.

14. The method as claimed in one of claims 11, 12 or  
15 13, characterized in that said gene encoding a selection agent is chosen from the bar gene which confers resistance to the herbicide Basta® and the NptII gene which confers resistance to kanamycin, said gene being included within the Ds transposable element.

20 15. An expression cassette comprising a fertility-restoring gene genetically linked to at least one gene encoding a "small seed" phenotype, combined with elements which allow their expression in plant cells,  
25 in particular a transcription promoter and terminator.

16. The expression cassette as claimed in claim 15, characterized in that said fertility-restoring gene is the barstar gene placed under the control of a promoter  
30 specific for pollen generation, in particular an anther-specific promoter such as pA3, pA6, pA9, pTA29, or of the Mac2 promoter, and of the CaMV 3' or Nos 3' terminator, genetically linked to a gene encoding a selection agent under the control of the actin  
35 promoter-actin intron and of the CaMV 3' or Nos 3' terminator.

17. The expression cassette as claimed in claim 15 or 16, characterized in that said gene encoding a "small

seed" phenotype is chosen from the shrunken 2 and brittle 2 genes in antisense orientation.

18. The expression cassette as claimed in any one of  
5 claims 15 to 17, characterized in that the promoter combined with the gene encoding a "small seed" phenotype is chosen from the HMWG and B32 promoters.

19. The expression cassette as claimed in any one of  
10 claims 15 to 18, characterized in that said terminator is chosen from the Nos 3' terminator and the CaMV 3' terminator.

20. A vector, in particular a plasmid, characterized  
15 in that it contains at least one expression cassette as described in one of claims 11 to 19.

21. A cellular host, in particular a bacterium such as *Agrobacterium tumefaciens* transformed with a vector as  
20 claimed in claim 20.

22. A maize cell transformed with at least one vector as claimed in claim 20.

23. A fertility-restoring young maize plant,  
25 characterized in that it comprises in its genome a fertility-restoring gene linked to a "small seed" phenotype marker.

24. A young maize plant homozygous for an AMS transgene and heterozygous for a fertility-restoring gene linked to a "small seed" phenotype marker, obtained from a seed as claimed in claim 3.  
30

25. A method for the multiplication of a young maize plant homozygous for an AMS transgene and heterozygous for a fertility-restoring gene linked to a "small seed" phenotype marker, comprising the steps consisting in:  
35

- 5 a) self-fertilizing young maize plants homozygous for an AMS transgene and heterozygous for a fertility-restoring gene linked to a "small seed" phenotype marker, which can be obtained by the method as claimed in either of claims 1 and 2,
- 10 b) selecting seeds homozygous for the AMS transgene and having a "small seed" phenotype,
- 15 c) selecting the seeds homozygous for the AMS transgene and heterozygous for a fertility-restoring gene linked to a "small seed" phenotype marker, obtained by self-fertilization of the young maize plants obtained from the seeds obtained according to step b).
- 20 26. The method as claimed in claim 25, characterized in that step b) comprises densimetric separation.
- 25 27. A kit for implementing the method as claimed in claim 25 or 26, characterized in that it comprises maize seeds homozygous for an AMS transgene and heterozygous for a fertility-restoring gene linked to a "small seed" phenotype marker, and oligonucleotides specific for the AMS transgene that are useful as primers for detecting, by PCR, the seeds homozygous for an AMS transgene and heterozygous for a fertility-restoring gene linked to a "small seed" phenotype marker.
- 30